Original Article

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T-786C variation in the promoter sequence of human *eNOS* gene markedly influences its expression level

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Summary **This study investigated the role of the T-786C polymorphism (SNP) in the 5'-flanking sequence of the endothelial nitric oxide synthase gene (*eNOS*) on its expression level in vascular endothelium with the ultimate goal of shedding more light on the mechanisms by which genetic variations of *eNOS* might affect the vascular level of nitric oxide (NO). Sequences in the 5'-flanking region of *eNOS* gene were PCR-amplified using genomic DNA templates isolated from blood samples collected from cardiovascular disease (CVD) patients. Two sequence-versions carrying the same SNP site were used; a short (345 bp) and an extended one (1,594 bp), numbered relative to the translational start site. All sequences were cloned into a promoter-less vector (pGL3-basic), which carries the firefly luciferase gene as a reporter. Genotyping of the T-786C polymorphism was performed using Sanger sequencing of the insert region. Luminescence levels were then recorded 24-48 h after transfecting human endothelial cell line (EA.hy926). Three genotypes were identified in the subject samples; TT, TC, or CC. The highest expression levels associated with the TT genotype, followed by the TC genotype, then the CC genotype. The extended sequence version produced higher expression levels compared to the shorter version. Our results provide evidence that the T allele at the T-786C SNP site of the *eNOS* gene results in increased expression of the enzyme, and consequently might provide a protective mechanism from CVD. The extended promoter sequence of *eNOS* resulted in higher expression of the gene, suggesting the presence of some essential binding sites for transcription enhancing proteins.**

Keywords: T-786C polymorphism, *eNOS* expression, luciferase, reporter gene

1. Introduction

Nitric oxide (NO) is a soluble gas produced in the vascular endothelium from L-arginine by the constitutive calcium-calmodulin-dependent enzyme endothelial nitric oxide synthase (eNOS). NO plays a crucial role in retaining the normal endothelium function and maintaining vascular homeostasis. NO maintains the vasodilatation tone of the vascular endothelium, regulates its cell growth, and protects it from injuries caused by platelet aggregation (*1*). Previous investigations on the production of endogenous NO suggested that vascular endothelium diseases probably result from reduced production of endogenous NO. Several factors are known to affect the release of endothelium NO including: down-regulation of eNOS, increased bioavailability of Asymmetric dimethylarginine (ADMA) that down-regulates the activity of eNOS, decreased bioavailability of Tetrahydrobiopterin (BH4), which is an essential co-factor for NOS activity, and the availability of reactive oxygen species (ROS), *e.g.* superoxide (O^{2-}) (*2,3*).

The *eNOS* gene (located on chromosome 7 at the 7q36.1 region) contains 26 exons and 25 introns spanning ~23.5 kb of genomic DNA. Although the gene is known to encode an mRNA of 4,052 nucleotides, some 10 splice variants of the gene have been reported (The Gene Cards human gene database index, ensemble). The gene has been the focus of intensive research to identify potentially functional polymorphisms or mutations that might affect mRNA

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transcription and gene expression. To date more than 1,730 polymorphic sites have been identified in, or in the vicinity of the gene (*http://www.ncbi.nlm.nih.gov/snp*). However, no clear correlation could be detected between the presence of SNPs in the coding and/or non-coding (*e.g*. promoter) sequences of the *eNOS* gene and NO production, despite the fact that several SNPs have been associated with cardiovascular disease (CVD) (*4*).

One of the most studied clinically relevant genetic variants is a SNP in the promoter region (T-786C; rs2070744), frequently associated with the development of coronary artery disease (CAD) (*5*). A statistically significant decrease in average eNOS expression has been reported for the T-786C polymorphism and was suggested to contribute to the vascular, contractile, and autonomic responses in failing human myocardium (*6*). Another study provided evidence that the same SNP is associated with the presence and severity of angiographically defined CAD in the Italian population (*7*).

We have previously investigated the distribution of several reported SNPs in a number of genes involved in the NO signaling pathway, including DDAH2 SNP1 (−1151 C/A, rs805304) and SNP2 (−449 C/G, rs805305) (*8*), eNOS (Glu298Asp polymorphism) (*9*), NADPH oxidase (C242T polymorphic site of p22 phox gene) (*10*) and paraoxonase (PON1 Q192R) (*11*), in a large number of DNA samples extracted from Egyptian healthy subjects and myocardial infarction (MI) patients. In these studies, however, no clear correlation could be detected between the reported SNPs and other relevant biochemical parameters.

Thus, the main goal of the present study is to investigate the effect of the T-786C polymorphism in the 5'-flanking promoter region of the *eNOS* gene on the gene expression level in the vascular endothelium, using a sample of Egyptian cardiovascular patients. To accomplish this goal, promoter sequences of *eNOS* carrying three reported SNPs were extracted from Egyptian cardiovascular patients before being cloned into a promoter-less vector (pGL3-basic) carrying the luciferase gene as a reporter. Luminescence levels were then recorded after transfecting human endothelial cell line (EA.hy926).

2. Materials and Methods

2.1. *Samples*

The T-786C SNP was examined in blood samples collected from acute myocardial infarction patients recruited for the study from the intensive care unit of the National Heart Institute, Imbaba, Giza and El Demerdash Hospital, Cairo, Egypt. Written informed consent were obtained from all participants in the study. The study protocol was approved by the German University in Cairo ethics committee and complied with Declaration of Helsinki. The main target is to classify the samples into TT, TC, or CC according to the genotype distribution of the *eNOS* T-786C SNP.

Blood samples were collected into EDTA coated vacuum tubes and stored at 4°C until DNA extractions were performed. Extracted DNA was used as a template for PCR amplification of the *eNOS* gene.

2.2. *Purification and amplification of DNA by polymerase chain reaction (PCR)*

DNA purification was done using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit (Rockford, USA). The purified DNA was used directly in the PCR. For PCR amplification of DNA, oligonucleotide primers were designed to flank either short or extended fragments upstream of the translation start site. An extended fragment represents the complete *eNOS* promoter (1,594 bp), while the short fragment (345 bp) is a basal central part of the promoter. Recognition sequences for restriction endonucleases were inserted at both ends of the corresponding oligonucleotide (Table 1). All PCR amplifications mixtures were carried out in a total volume of 100 µL containing 50 µL EmeraldAmp GT PCR Master Mix (TAKARA, Japan), 2 µg genomic DNA as a template, 40 pmol final concentrations from each of the forward and reverse primers, and the volume was completed to 100 µL using nuclease-free sterile water. The PCR amplification programs for the long and short fragments are listed in Tables 2 and 3, respectively. The amplified PCR products were purified using PureLink PCR Purification Kit (Thermo Fisher

Table 1. Primer sequences

Primer name	Sequence (Underlined Restriction Sites)	Amplicon size
Forward *eNOS* primer – extended insert	GAAGATCTATCTGATGCTGCCTGTCACCTTGACCCTGAG (*Bgl* II)	1,612bp
Reverse *eNOS* primer – extended insert	ATTAAGCTTTGCCTGCTCCAGCAGAGCCCTGGCCTTTTC (*Hind* III)	
Forward *eNOS* primer – extended insert	GAAGATCTCTGATGCTGC CTGTCACCTT (*Bgl* II)	1,594bp
Reverse *eNOS* primer – extended insert	ATTAAGCTTCCAGCAGAG CCCTGGCCTT (*Hind* III)	
Forward *eNOS* primer – short insert (primers use for sequencing)	GAAGATCTAGATGGC ACAGAACTACAAACC (*Bgl* II)	345bp
Reverse *eNOS* primer – short (primers use for sequencing)	ATTAAGCTTGTCCTT GAGTCTGACATTAGG (*Hind* III)	

Table 2. PCR thermo-cycling conditions for the long insert (1,612 bp)

Protocol step	Temperature	Time	Number of cycles
Initial step	95°C	10 min	1
Denaturation step	95°C	30 sec	35
Annealing step	60°C	30 sec	
Extension step	72°C	2 min	
Final elongation	72°C	10 min	1
Final hold	4°C	Pause	hold

Table 3. PCR thermo-cycling conditions for the short insert (345 bp)

Protocol step	Temperature	Time	Number of cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing	57°C	30 sec	
Extension	72°C	1 min	
Final elongation	72°C	10 min	1
Pause	4°C	Hold	

Scientific, Rockford, USA) and their concentrations estimated using Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific). The amplified fragments were then digested using FastDigest *Bgl* II and *Hin*d III (Thermo Fisher Scientific, Rockford, USA) before being ligated into a pGL3-basic vector (Promega, Madison, USA); Luciferase reporter vector (E1741, Promega, USA) previously restricted using the same endonucleases. After an overnight incubation at 23°C, the ligation mixtures were transformed into competent *E. coli* XL1 cells (Stratagene, USA). All recombinant plasmid sequences were confirmed by Sanger chain termination sequencing.

2.3. *Transfection of EA.hy926 cells using the polyethylenimine (PEI) reagent*

Procedures were done following the protocols of a previous study (*12*). Basically 600 ng of plasmid DNA (*e-NOS* TT SNP, *e-NOS* TC SNP, *e-NOS* CC SNP long, or *e-NOS* CC SNP short, or the control plasmids pGL3-basic or pSV-β-galactosidase) were added to 1.8 µg of PEI reagent and completed to 40 µL using Dulbecco's Modified Eagle's Medium (DMEM) without serum. Transfection mixtures were incubated at room temperature for 15 minutes. Each transfection mixture (40 µL) was transferred to 24-well plate previously seeded with 10^5 cells/well; where each well contained 400 µL of total liquid volume (360 µL of complete medium and 40 µL of transfection mixture). The plate was incubated at 37°C for 48 hours.

Cell lysis and luminescence assays were performed using 1× Glo Lysis Buffer, Steady-Glo, and Beta-Glo Luciferase Assay Systems (Promega, Madison, USA). Luminescence was measured using GloMax®-Multi Detection System (Promega, Madison, USA).

2.4. *Statistical analysis*

Average luminescence levels were determined for three independent measurements and differences were analyzed using analysis of variance (ANOVA) using GraphPad Prism v5.

3. Results

3.1. *Effect of T-786C SNP on luciferase expression using pGL3 eNOS promoters*

EA.hy926 cells were transfected with plasmid constructs carrying the different promoter fragments. pSV-β-galactosidase was co-transfected into the cells as a control for transfection efficiency. Both luminescence intensity and β-galactosidase activities were measured in cell lysates 48 hours post-transfection. Luminescence levels were normalized to β-galactosidase activities. Luminescence levels for EA.hy926 cells harboring the CC_{short}-786 *eNOS* promoter constructs were not significantly different ($p = 0.5903$) from those obtained for cells transfected with empty plasmid vectors. On the other hand, the CT_{short} and TT_{short} SNP genotypes resulted in luminescence levels that were 1.6 and 1.8-fold higher than their respective controls ($p < 0.0001$). Luminescence results obtained for constructs carrying CC_{short}-786, CT_{short}, TT_{short} were significantly different from each other's as shown in Figure 1.

3.2. *Effect of eNOS promoter sequence length on luciferase expression*

Luminescence results obtained for EA.hy926 cells transfected with constructs carrying the extended version of *eNOS* promoter ($eNOS_{long}$) with the CC_{-786} genotype

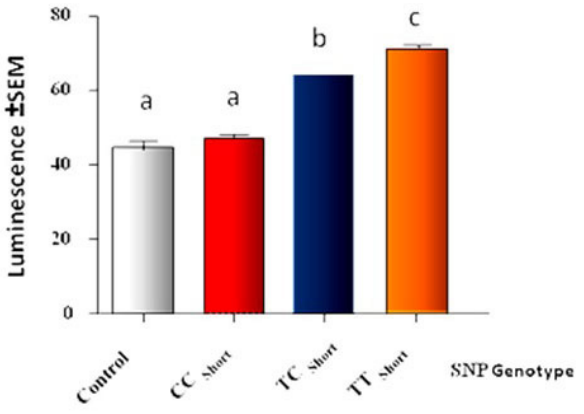

Figure 1. Comparison between luminescence levels obtained for EA.hy926 cells transfected with plasmid constructs of short *eNOS* promoter sequences carrying the three different SNPs. Control luminescence is the Luminescence produced by EA.hy926 cells transfected with the empty plasmid vector pGL3-Basic. Differences in luminescence emission were analyzed using independent *t* test, where different letters (a, b, or c on bars) indicate significant differences at ***$p < 0.0001$. Error bars represent standard error of the mean (SEM).

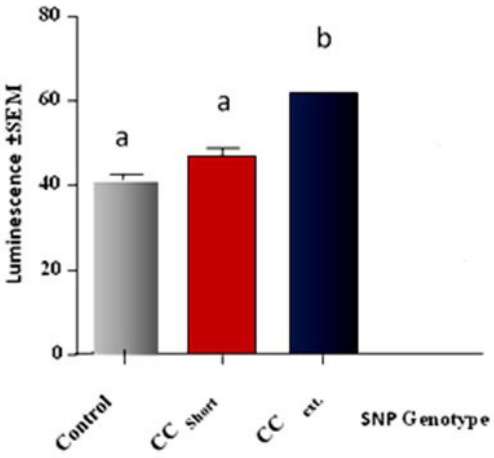

Figure 2. Luminescence levels obtained using constructs carrying the CC genotype on the entire, or shorter *eNOS* promoter sequences. Control luminescence is the Luminescence produced by EA.hy926 cells transfected with the empty plasmid vector pGL3-Basic. Differences in luminescence emission were analyzed using independent t test, where different letters indicate significant differences at $^{***}p <$ 0.0001. Error bars represent standard error of the mean (SEM).

(CC_{long} = 1,594 bp) were significantly higher than those obtained for cells transfected with constructs of the shorter promoter version (CC_{short}) that carries the same SNP site (Figure 2).

4. Discussion

To date more than 1,730 polymorphic sites have been identified in, or in the vicinity of the *eNOS* gene (*http://www.ncbi.nlm.nih.gov/snp*). However, no clear correlation could be detected between the presence of SNPs in the coding or non-coding sequences of the gene and the amount of NO produced to reflect the activity of the encoded enzyme.

Here, we extended our studies on *eNOS* by performing *in vitro* gene expression analyses of the T-786C polymorphism in the promoter region of the *eNOS* gene, with the ultimate goal of shedding more light on the mechanisms by which genetic variations of *eNOS* might affect the expression of eNOS and therefore the vascular level of NO.

DNA sequencing of amplified regions of the *eNOS* gene revealed the presence of three different genotypes; T/T, T/C, and C/C. Our results show significantly higher expression levels of *eNOS* for the T/T genotype compared to the other two genotypes; a T to C mutation resulted in 1.7~1.9-fold lower expression levels of the gene. In agreement with our findings, earlier *in vitro* observations suggested that the replacement of a T at position – 786 by a C resulted in ~50% lower transcriptional activity (*13,14*).

In harmony, Cattaruzza *et al*. (2004) investigated the effect of T-786C SNP on the shear stress-induced NO release from endothelial cells isolated from umbilical cords. They found that shear stress-induced *eNOS* mRNA and protein expression were present in TT and CT genotype cells but absent in cells with CC genotype. They concluded that the T-786C SNP of *eNOS* gene constitutes a genetic risk factor for CAD, presumably due to binding of an inhibitory transcription factor to the C-type promoter blocking shear stress-dependent maintenance of *eNOS* expression (*15*).

In their trials to elucidate the molecular mechanism behind the reduced *eNOS* gene expression associated with the T-786C mutation, Miyamoto *et al*. were able to identify and purify a protein called replication protein A1 (RPA1) that contributes to the diminished *eNOS* expression in T-786C placenta cells. Diminished *eNOS* expression was revealed by the finding that serum nitrite/nitrate levels among individuals carrying the T-786C SNP were significantly lower than among the T variant subjects. They concluded that RPA1 apparently functions as a repressor protein in the T-786C polymorphism-related reduction of *eNOS* gene transcription associated with the development of CAD (*14*).

AliReza *et al*. investigated the frequency of T-786C polymorphism of the *eNOS* gene in non-smoker and non-diabetic CAD patients in North West Iran. Compared to the healthy control group, the *eNOS* T-786C genotype was associated with reduced serum levels of NO (*16*). In a sample of Japanese population, Nakayama *et al*. assessed the influence of the same polymorphism on coronary spasm development. They found that the T-786C mutation resulted in a significant reduction in *eNOS* gene promoter activity that reduces the endothelial NO synthesis and predisposes the patients to coronary spasm (*13*). Moreover, Popov *et al.* demonstrated that T-786C polymorphism contributes to a higher prevalence of postoperative mortality after emergency cardiac surgery. Thus, the *eNOS* T-786C polymorphism could serve to differentiate high risk subgroups in individuals with cardiac disease who need cardiac surgery with cardiopulmonary bypass (*17*).

In contrast, however, Kincl *et al*. found no impact of T-786C polymorphism on CAD prognosis (death or AMI, unstable angina, necessity of percutaneous or coronary artery bypass graft, heart failure, or cardioverter/defibrillator implantation) (*18*). Similarly, Jaramillo *et al*. and Alp *et al*. observed that T-786C polymorphism of the *eNOS* gene was not associated with CAD in a studied sample of Chilean and Turkish individuals, respectively (*19,20*). To our knowledge, no parallel investigations were performed on Egyptian population regarding the association of T-786C polymorphism of *eNOS* with the incidence of CAD.

Two stretches of *eNOS* promoter were tested in the study; a short sequence (345 bp) carrying the CC genotype and a longer promoter sequence (1,594 bp) carrying the same genotype. Luminescence levels obtained for EA.hy926 cells transfected with constructs carrying the longer promoter version were significantly higher than those obtained from cells bearing the shorter

inserts ($p < 0.0001$).

A study made by Xing *et al*. using DNA sequence deletion, concluded that 68% of the basal activity of the *eNOS* promoter was controlled by the region from – 1 to – 166 bp and the rest was dependent on the region from – 1,033 to – 1,600 bp (*21*). The study strongly suggested that the main functional region of *eNOS* promoter is from – 1 to – 166 bp that binds to the upstream activator transcription factor 1 (AP1). This explains the increased gene expression using the extended sequence for *eNOS* promoter (long version = 1,594 bp) observed in our study as compared to the gene expression using the short version (345 bp) that lacks the binding site to transcription factor AP1.

In conclusion, our study suggests that a T allele at the – 786 site of *eNOS* gene increases genetic expression of the enzyme, while the CC genotype at the same site could be a predisposing factor to CVD through marked down expression of *eNOS*.

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